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Original Article

Neuroprotective effect of esmolol in a porcine model of cardiac arrest

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Abstract: Protection of the neurological function in post cardiac arrest (CA) patients is still a challenge. Given that cardiac function of post-CA patients can be improved by β-blocker, namely esmolol. This study examined the effects of esmolol on post-resuscitation induced impairment of nerve function.Ventricular fibrillation (VF) was first induced in pigs (n=24). After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard cardiac pulmonary resuscitation (CPR) and defibrillation. Cerebral performance categories (CPC) score was assessed in living pigs. Blood samples and brain tissues were collected for subsequent immunohistochemistry, western blotting, and histopathological examinations, as well as tissue electron microscopy. Esmolol decreased CPC score, improved nerve function, and reduced cerebral edema, as compared with epinephrine only or normal saline treatments. These effects were associated with decreased serum levels of HIF-1α, VEGF, and VEGFR1, and lower Ang-2/Ang-1 in early return of spontaneous circulation (ROSC), and then affects the express in the brain. Smolol relieved the animals' neurologic impairment after ROSC. This might be due to its inhibitory effects on HIF-1α and VEGF systems in early restoration.

Keywords: Esmolol, cardiac arrest, neurological impairment, BBB, HIF-1α, VEGF, Ang-1

Introduction

Almost 326,200 people have an onset of sudden cardiac arrest annually in America. The survival rate of out-of-hospital cardiac arrest is only 10.6%, with only 8.3% of survivors whose nerve function is not impaired. Patients with nerve function impairment after sudden cardiac arrest have brought a huge burden to their families and society [1].

Epinephrine has been used for cardiac pulmonary resuscitation (CPR) for many years. It is universally believed that it can improve the success rate of CPR, as it can activate the α-adrenergic receptor, and increase coronary perfusion pressure through systemic arteriolar vasoconstriction, as well as maintain peripheral vascular tone and prevent arteriolar collapse [2]. This may contribute to the effects of epinephrine on increasing the rate of return of spontaneous circulation (ROSC) in patients. Compared with placebos, epinephrine can promote patients' ROSC rate and survival rate in hospitalization without decreasing mortality of discharged patients [3]. However, previous studies have shown that epinephrine cannot attenuate post-resuscitation neurologic impairment [3, 4]. Administration of epinephrine to a cardiac arrest (CA) patient before arriving at the hospital may even induce serious neurologic impairment [5]. Administration of high dose epinephrine in out-of-hospital cardiac arrest patient can worsen neurologic impairment [6]. These effects of epinephrine may be due to the facts that epinephrine induces blood vessels contractions, resulting in reduced blood flow to brain, and leading to function impairment [3]. Many clinical studies have been conducted in attempts to improve the post-resusci-

tation organ function. It has been found that epinephrine can activate the β-receptor, thereby increasing myocardial oxygen consumption in ventricle fibrillation. As such, some researchers have proposed that administration of a β-receptor antagonist canimprove the effects of epinephrine in ventricle fibrillation and promoted the survival rate [7-9]. To support this hypothesis, it has been found that β-blockade can decrease neurologic damage in the ischemia brain [10-12].

While studies have shown that various factors might be involved in CA-induced neurological impairment, little is known about the effects of β-receptor antagonist on these regulatory factors. For example, the hypoxia-inducible factor (HIF-1α) is a regulatory factor in cells, which is critical to hypoxia response, and can regulate the transcription of multiple downstream genes due to cerebral hypoxia-ischemia after sudden cardiac arrest. Additionally, the vascular endothelial growth factor (VEGF) is related to revascularization and has the potential function of increasing vascular permeability. VEGFR1, one of its receptors, plays a very important role in regulating vascular permeability under the pathological conditions of inflammation and ischemia [13]. Also, the angiopoietin (Ang)-Tie2 ligand-receptor system is crucial in ischemia-induced neovascularization.

Therefore, we hypothesized that the combined administration of epinephrine and esmolol, a short-acting β-blocker, to patients with sudden CA can reduce nerve impairment. To this end, we established a cardiac arrest model in pigs. Ventricular fibrillation (VF) was first induced in animals. After 8 min of non-treatment operation, animals received a bolus of esmololand epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. We determined the change in nerve function by CPC score and serum neuron-specific enolase (NSE). Blood samples and brain tissues were collected for subsequent immunohistochemistry, western blotting, and histopathological examinations, as well as tissue electron microscopy.

Materials and methods

Animal surgical procedure

Our research has been approved by the Animal Care and Use Committee at the Chaoyang Hospital of Capital Medical University, China. We carried out our experiment in accordance with the guidelines for animal care. All surgeries were performed under anesthesia and analgesia, and every effort was made to minimize suffering. Twenty-four Beijing Chang Bai pigs at 12-14 months of age, weighing 35±5 kg were randomly divided into three groups (n=8/group), including the esmolol treatment group (S group; esmolol 500 μg/kg + epinephrine 20 μg/kg per 20 mL dilution, bolus injection), epinephrine treatment group (E group; epinephrine 20 μg/kg per 20 mL dilution, bolus injection), and saline treatment group (NS group; normal saline 20 mL, bolus injection). All animals received initial sedation with 10 mg/kg of intramuscular ketamine and 0.5 mg/kg of midazolam. The anesthesia was induced by propofol (1.0 mg/kg) through the ear vein and maintained with the intravenous infusion of pentobarbital (8 mg/kg/hour). Tracheotomy was performed and a cuffed 6.5-mm endotracheal tube was placed (Covidien, Covidien Ireland Limited, Ireland). The pigs were mechanically ventilated (evita 4, Dräger, Lübeck, Germany) with 30% oxygen in the air and volume-controlled mode after preparation. Mechanical ventilation was instituted with 5 cm H_2O positive end-expiratory pressure (PEEP) and minute volume set to maintain arterial $PaCO_2$ at an average of 5-5.5 kPa. The End-tidal PCO_2 ($ETCO_2$), blood oxygen saturation and electrocardiogram (Intelli Vue MP30, Philips, Boeblingen, Germany) were measured. A Picco catheter (Pulsion Medical Systems, Munich, Germany) was advanced into the left femoral artery, and the continued arterial pressure and cardiac output (CO) was measured. A 5-Fr pacing catheter was advanced from the right femoral vein into the right ventricle in order to induce ventricular fibrillation (VF). A 14-G saline-filled double lumen catheter (Arrow international Inc., NC, USA) was placed into the right atrium via right external jugular vein to measure the right atrial pressure and for drug administration.

Experimental treatment

We collected blood samples for baseline analysis and measured CO and other hemodynamic data after the stabilization of the animals. An electrical stimulator (GY-600A; Kaifeng Huanan Equipment Co., Ltd., Kaifeng, China) was connected to the temporary pacemaker conductor, which was inserted into the

right ventricle, and programmed in the S1S2 mode (300/200 ms), 40 V, 8:1 proportion, and 10-ms step length to provide continuous electrical stimulus until VF [14]. After VF, the pig was untreated for 8 minutes, and mechanical ventilation was weaned at the same time. Manual chest compression was initiated at a rate of 100 compressions per minute with equal compression and relaxation duration. The compression depth was equivalent to 30% of the anteroposterior diameter of the chest. Room air was inspired by a bag respirator. The compression-to-ventilation ratio was 30:2. The operators of CPR were members of our team who had been well trained. The quality of the chest compressions was controlled by the QCPR (Philips Medical Systems, Best, Holland). The CPR continued for 2 minutes, and at that time one observer opened a sealed envelope which contained instructions to assign the pig to one of the three groups. The S group received a bolus of esmolol and epinephrine (500 μg/kg and 20 μg/kg, respectively). The E group received a bolus of epinephrine (20 μg/kg) only. The NS group received a bolus of normal saline. The drugs were injected through the central line. The operators of CPR did not know the pigs' groups. After 2 minutes of CPR, defibrillation (SMART Biphasic) was performed with an energy of 120 J (about 4 J/kg). CPR was rapidly resumed if it failed to attain ROSC. The energy of the second defibrillation was diphase 150 J. If the normal rhythm of the heart was found through the ECG or arterial pressure wave form, the CPR was stopped. ROSC was defined as an arterial systolic pressure >60 mmHg for at least 10 minutes. The CPR was repeated for a maximum of 15 minutes. If ROSC was not achieved, the animal was announced as dead. Mechanical ventilation was used with 100% oxygen for ten minutes after ROSC, and then the concentration of oxygen was decreased to 30%. The surviving animals were monitored for 12 hours, with anesthesia maintained. The temporary pacemaker and pacing catheter were then removed. The pig was placed in a cage and observed for additional 12 hours. The porcine cerebral performance category was evaluated at 24 hours after ROSC. The animals were administered with anesthesia and monitored again after CPC score evaluation. We obtained blood samples, arterial blood gas analysis and PICCO parameter at 0.5, 6, 12 and 24 hours after ROSC. The pigs were euthanatized by intravenous bolus pentobarbital. Autopsy was performed and the brain was obtained. Corticocerebral specimens were taken and frozen in liquid nitrogen and stored at -80°C.

Brain water content

Brain tissue was immediately weighed in order to measure the wet weight. The tissues were dried in an oven (105°C) for 72 hours, and then the dry weight was measured. Brain water content (%) was calculated as (wet weight - dry weight)/wet weight × 100%.

Cerebral performance categories

The neurological outcome was evaluatedat 24 hours after ROSC using the pig cerebral performance categories (CPC) score, which used a five-point scale to access the neurological function. CPC 1 suggests normal neurological outcome: the animal has no difficulty in eating, standing, walking, drinking, remaining alert and fully responding to environmental stimuli. CPC 2 suggests mild neurologic impairment, with sufficient cerebral function for independent activities of daily life, and responding slowly to environmental stimuli. CPC 3 suggests severe cerebral disability: the animal is unable to conduct daily support, and does not respond normally to environmental stimuli. CPC 4 suggests coma or vegetative state without interaction with the environment. CPC 5 suggests brain death. CPC 1 and CPC 2 were considered to be good neurological outcomes. Animal treatment and grouping information was blinded to experimenter who evaluated CPC according to methods previously described [15].

Enzyme linked immunosorbent assay (ELISA)

We measured serum NSE (abcam, Shanghai, China) to predict neurologic outcome. HIF-1α (abccam, China), VEGF (bioss, China), VEGFR-1 (bioss, China), VEGFR-2 (bioss, China), Ang-1 (bioss, China), Ang-2 (bioss, China), and Tie-2 (bioss, China) were also measured at the same time.

Immunohistochemistry (IHC) and histology assay

The pig corticocerebraltissue was processed routinely for preparation of paraffin-embedded blocks. 4 mm-thick serial cross sections were collected from each tissue block, then were deparaffinized and hydrated in xylene in a graded alcohol series. We used high pressure treat-

Table 1. Baseline data

	E group (n=8)	S group (n=8)	NS group (n=8)	p
Weight (kg)	34.06±1.63	33.3±2.22	33.39±2.05	0.706
HR	111.63±12.00	109.75±19.45	108.25±14.19	0.910
MAP (mmHg)	95.75±5.73	102.13±9.45	98.88±9.17	0.866
CO (L/min)	4.20±0.49	4.06±0.36	4.18±0.48	0.801
EVLW (ml/kg)	7.14±1.73	6.50±1.60	6.50±1.51	0.667

ment in citrate-buffer (10 mM, pH=6.0) for 15 minutes to perform antigen retrieval, then washed the section with phosphate-buffered saline (PBS; 20 mM, PH=7.4). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide/methanol. The primary antibody and dilution used in IHC were NSE (Abcam, Shanghai, China), VEGF (Abcam, Shanghai, China), VEGFR-1 (Abcam, Shanghai, China), VEGFR-2 (Abcam, Shanghai, China), HIF1-α (Abcam, Shanghai, China), Ang-1 (Abcam, Shanghai, China), Ang-2 (Abcam, Shanghai, China), and Tie-2 (Abcam, Shanghai, China). The section was then stained with haematoxylin, and Image J software (National Institutes of Health, Bethesda, MD, USA) was used to determine the optical density of the images after the second incubation. Five sections were selected from each group, and the average optical density of five high-power fields (× 400) from each slice was measured. Each sample was tested in triplicate. The histology slides were stained with hematoxylin and eosin (H&E).

Western blot

20-mg frozen brain sample was homogenized in 2 mL of ice-cold buffer comprising 50 mM Tris HCl (pH=7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthova-nadate, sodium fluoride, EDTA, leptin, and PMSF (final concentration was 1 mM). The samples were centrifuged for 25 min at 15,000 r/min at 4°C. The supernatant containing total protein was collected, and the protein concentration was determined by the Bradford method. A total of 40 microgram proteins were separated on 10% or 12% polyacrylamide gels, and were transferred onto polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies: HIF-1α (1:5000; Abcam, Shanghai, China), VEGF (1:10000; Abcam, Shanghai, China), or Ang1 (1:10000; Abcam, Shanghai, China), at 4°C overnight or for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized on film and scanned. The densities of the protein blots were analyzed using Lab works Software (Ultra-Violet Products Ltd., Cambridge, UK) and normalized to β-actin levels.

Transmission electron microscopy

Brain tissue was preserved using 4% paraformaldehyde to assess pathological and ultrastructural changes of the brain under transmission electron microscope (TEM; H-7650; Hitachi, Tokyo, Japan).

Statistical analyses

Data was expressed as mean ± standard deviation (SD). The data was analyzed using analysis of variance (ANOVA) followed by least significant difference (LSD) test. In addition, the continuous variables were fixed to normal distribution and equal variances by the Kolmogorov-Smirnov test and homogeneity of the variance test. The ratio of outcome between groups was performed by a Chi-square analysis. Statistical significance was defined as $P < 0.05$. All data analyses were conducted with the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Esmololdid not change the resuscitation rate

The basic status of the three groups of animals did not show difference (**Table 1**). A total of five animals died. The E group had a survival rate of 87% at 24 hours, the S group 87.5% and the NS group 62.5%, which is the lowest. However, no significant difference in mortality was achieved. In contrast, the NS group hadthe largest number of defibrillation times ($P<0.05$) with the longest time for ROSC ($P<0.01$; **Tables 2**, **3**).

Esmolol group had good neurological outcome

The S group had a favorable nerve function assessment ratio (CPC score of 1 or 2) of 87.5% (**Table 3**), which was higher than those of E group and the NS group. The levels of serum NSE gradually increased after ROSC. However,

Table 2. Resuscitation Outcome

	E group (n=8)	S group (n=8)	NS group (n=8)	E vs S	E vs NS	S vs NS
Time to ROSC (s)	200.43±55.06	180.57±58.65	402.00±133.12$^{**,\#\#}$	P=0.66	P=0.001	P=0.000
Number of shock	2.12±2.03	2.00±2.07	4.50±2.27$^{*,\#}$	P=0.907	P=0.036	P=0.028

E VS NS *= P<0.05 **= P<0.01; S VS NS #= P<0.05 ##= P<0.01; E VS S $= P<0.05 $$=P<0.01.

Table 3. neurological outcome and survival

	E group (n=8)	S group (n=8)	NS group (n=8)	p
24 Hsurvival	7	7	5	0.383
CPC=1, 2	3	7	4	0.03
CPC=1	1	6	2	0.005

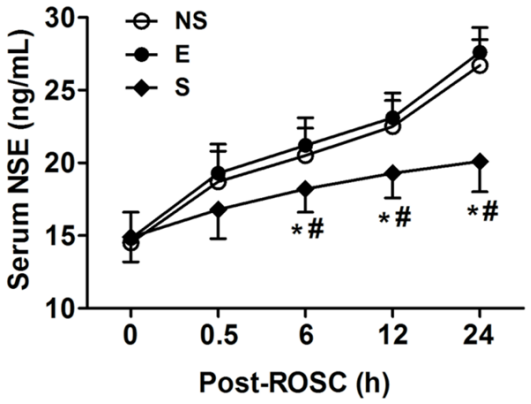

Figure 1. The levels of serum NSE. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group (P<0.05). *Pond* represents significant difference, as compared to E group (P<0.05).

the level of serum NSE in the S group was lower than the E and NS groups, at 6, 12, and 24 hours after ROSC (P<0.05; **Figure 1**).

Esmolol decreased the brain water content

The brain water content of the three groups of animals' cerebral tissue was different. Specifically, the S group showed the lowest brain water content, which had significant significance, as compared with the E and NS groups (P<0.05; **Figure 2**).

Esmolol affect the serum Ang/Tie system and HIF/VEGF system

The level of serum Ang-1 in the S group rose immediately after ROSC, and was higher

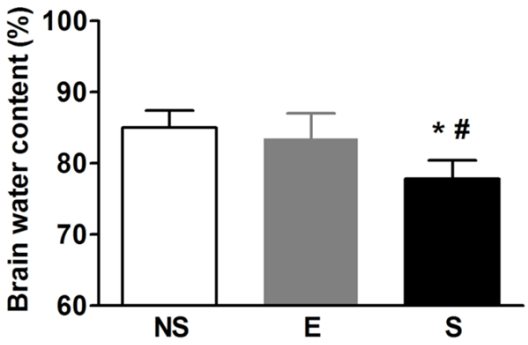

Figure 2. The brain water content of the three groups of animals. Brain water content (%) was calculated as (wet weight - dry weight)/wet weight × 100%. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg and 20 μg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group (P<0.05). *Pond* represents significant difference, as compared to E group (P<0.05).

thanthe level of serum Ang-1 in the E and NS groups of animals (P<0.05; **Figure 3**). The level of serum Ang-2 remained same across time of ROSC. We also calculated the ratio of Ang-2/Ang-1, and found that Ang-2/Ang-1 in the S group was lower than the NS and E group at 0.5 hour (P=0.004) and 24 hours (P=0.017) after ROSC. Furthermore, the serum level of angiogenic factors receptor Tie-2 was higher in the S group than that in the NS group at 0.5 hours (P=0.016; **Figure 3**).

The HIF-1α level in the serum increased after ROSC, but animals in the S group showed a slower increase. Compared with the E and NS group, HIF-1α level in the S group was lower at 0.5 hours, 6 hours (P=0.023), and 12 hours (P=0.049) after ROSC (**Figure 4**). However, HIF-1α level in the S group rose higher at 24 hours after ROSC (P=0.007; **Figure 4**), as compared with the E group.

The serum level of VEGF exhibited a similar trend as that of HIF-1α levels. The serum level of VEGF in the E group was remarkably higher

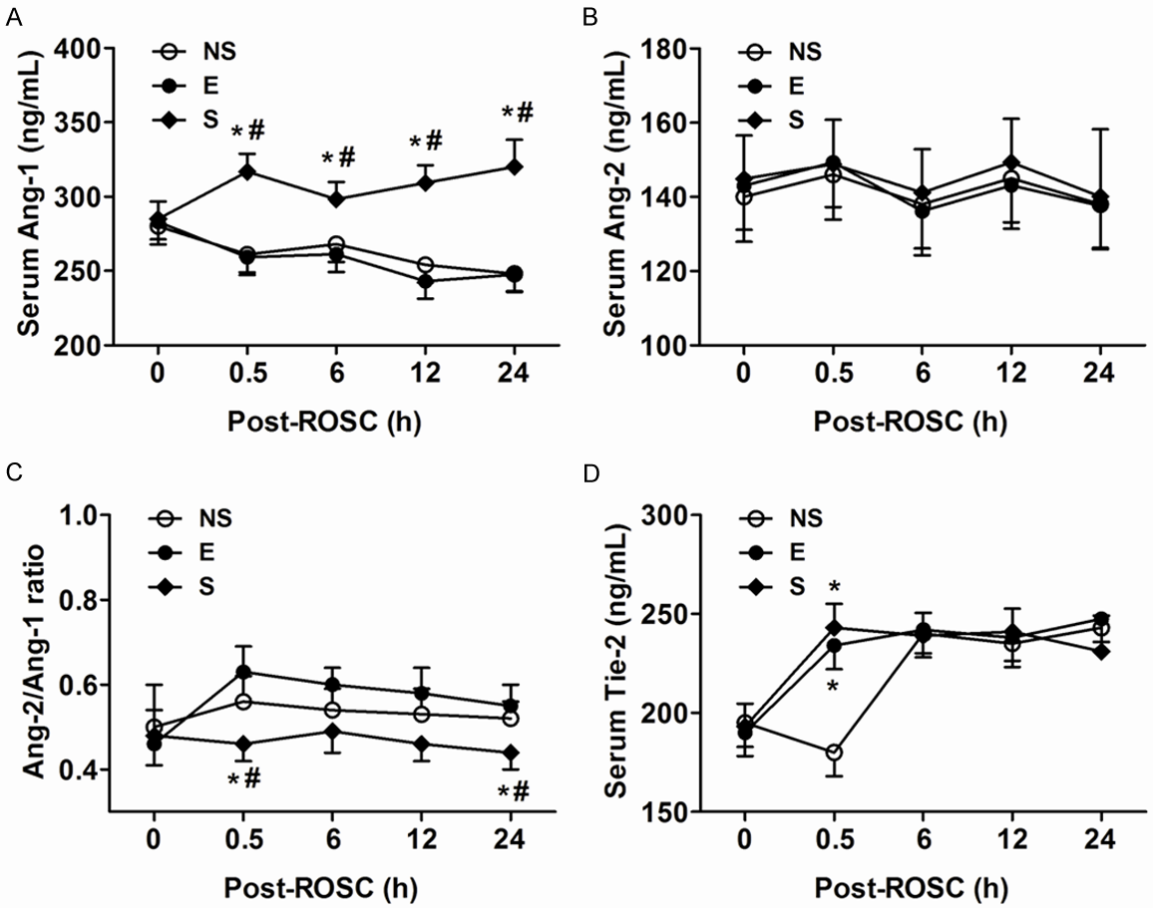

Figure 3. The post-ROSC effects of AC on Ang-Tie2 system. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group (P<0.05). *Pond* represents significant difference, as compared to E group (P<0.05).

than the S group at 0.5 hours after ROSC (P=0.0034; **Figure 4**), but lower than the S group at 24 hours after ROSC (P=0.046; **Figure 4**). The serum level of VEGFR1 decreased robustly at 0.5 hours after ROSC, as compared with that of the E and NS groups (P<0.001; **Figure 4**). The serum level of VEGFR1 in the S group was also lower than the NS and E group at 6 and 12 hours after ROSC (P<0.05; **Figure 4**). However, the serum level of VEGFR1 in the NS group was lower than the other two groups at the 24 hours after ROSC (P<0.05; **Figure 4**). The serum level of VEGFR2 in the S group was lower than the NS and E group at 12 hours after ROSC (P<0.05; **Figure 4**). However, the serum level of VEGFR2 in the NS group was lower than the other two groups at the 24 hours after ROSC (P<0.05; **Figure 4**).

The protein expressed in the cortex at 24 h after ROSC

We found that the expression of Ang-1 in the cerebral cortex in the S group was higher than that of the E and NS groups (P<0.001; **Figure 5**). The expression of Ang-1 in the cerebral cortex in the NS group was higher than the E group (P<0.05; **Figure 5**). However, the expression of Ang-2 in the S group was lower than that of the E and NS groups (P<0.001; **Figure 5**). The expression of Tie-2 in the S group was higher than that of the E and NS groups (P<0.01; **Figure 5**).

Additionally, we found that the expression HIF-1α in the S group was higher than the E and NS groups (P<0.001; **Figure 6**). The expression HIF-1α in the NS group was higher than the E

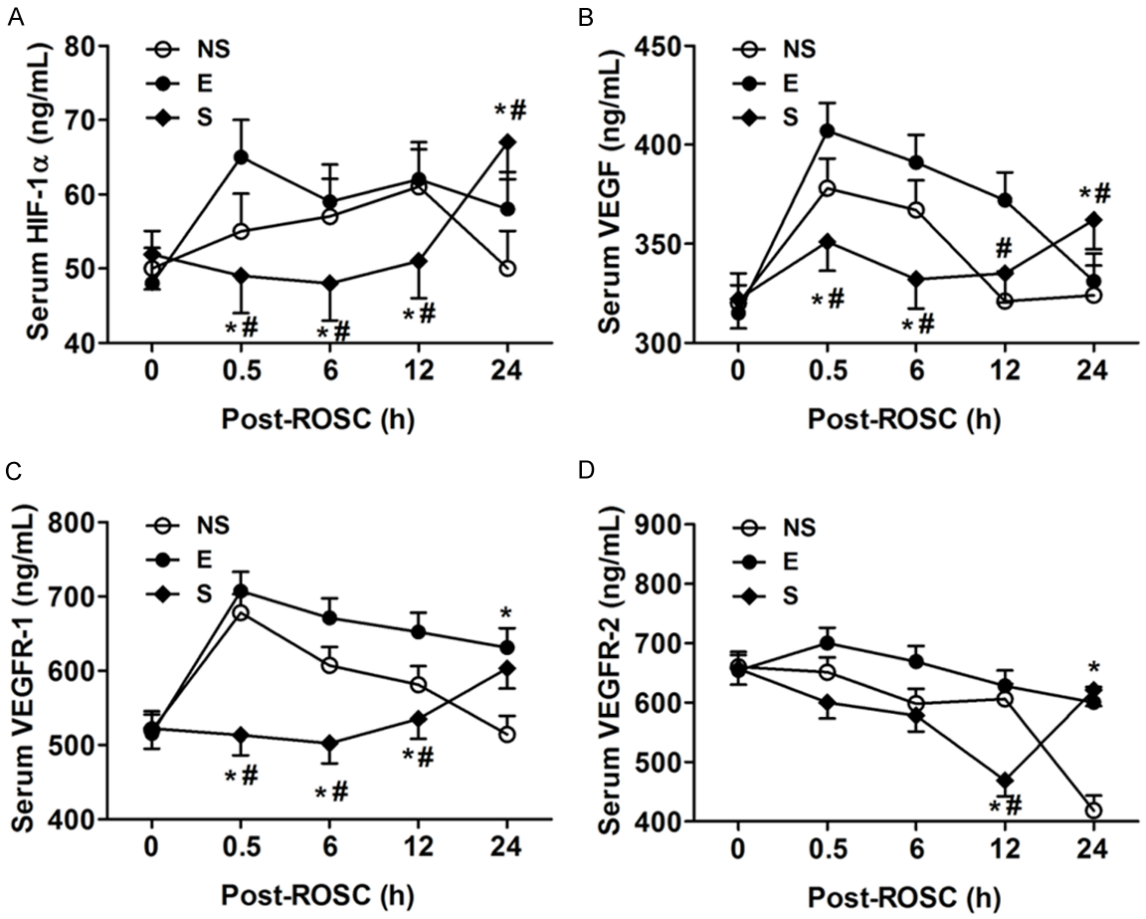

Figure 4. The post-ROSC effects of AC on HIF-1α, VEGF, VEGFR1, and VEGFR2. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group (P<0.05). *Pond* represents significant difference, as compared to E group (P<0.05).

group (P<0.001; **Figure 6**). The result of VEGF expression was similar to that of HIF-1α. Namely, the expression of VEGF in the S group was higher than the E and NS groups (P< 0.001; **Figure 6**), and the expression of VEGF in the NS group was higher than the E group (P<0.001; **Figure 6**). The result of VEGFR-1 expression was identical to that of VEGF. Namely, the expression of VEGFR-1 in the S group was higher than the E and NS groups (P<0.001; **Figure 6**), and the expression of VEGFR-1 in the NS group was higher than the E group (P<0.001; **Figure 6**). The expression of VEGFR-2 in the E group was higher than the NS groups (P<0.001; **Figure 6**), and the expression of VEGFR-2 in the S group was lower than the E and NS group (P<0.001; **Figure 6**).

Western blot was conducted to verify immunohistochemical results. We found that the protein levels of Ang-1, HIF-1α, and VEGF in the S group were higher than the E and NS groups (P<0.005; **Figure 7**).

Esmolol decreased histopathologic changes

We found that the gross morphology of cells in the brain tissues was altered after AC. In general, normal cell morphology was lost in brain tissues of animals after AC. Cells exhibited necrocytosis under the electron microscope due to ischemia and anoxia after cardiac arrest. The E group's animals had the worst situation. The NS group's animals exhibited less necrocytosis in brain cells. The S group's animals showed integral cells with little damage (**Figure 8**).

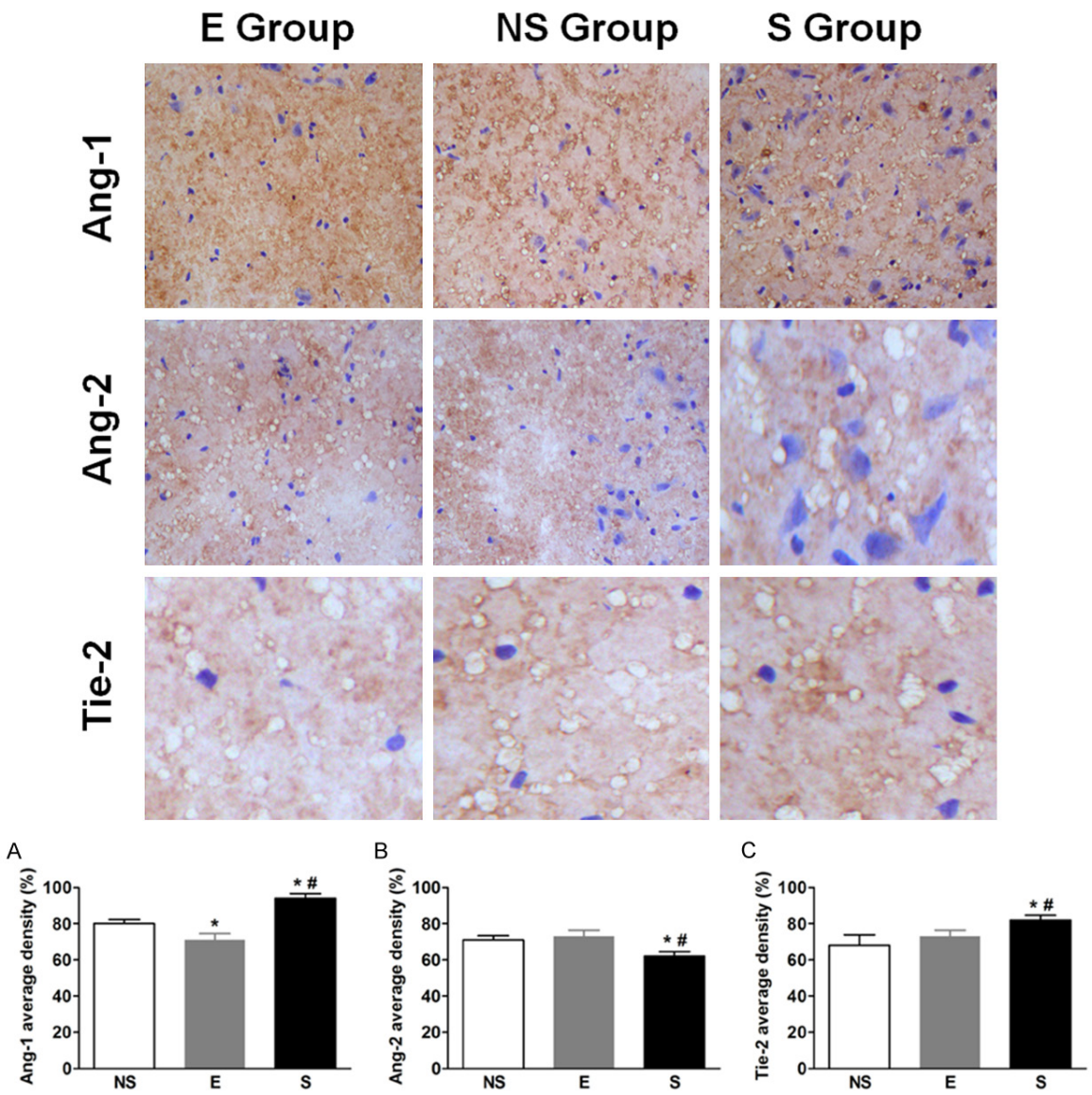

Figure 5. The post-ROSC effects of AC on the protein expression of Ang-Tie2 system using immunohistochemistry methods. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group (P<0.05). *Pond* represents significant difference, as compared to E group (P<0.05).

Esmololpreserved ultrastructural morphology

We observed the ultrastructure through a TEM and found that the blood brain barrier (BBB) was damaged, and astrocytes exhibited cellular edema, structural damage, and air bubbles. Regarding the degree of severity, the E group was the worst, the NS group was less, and the S group was the slightest, with less damage and relatively integral mitochondria structures in cells (**Figure 9**).

Discussion

We found in this study that animals with the combined treatment of esmolol and epinephrine after cardiac arrest had slighter nerve injuries, as compared with epinephrine alone or saline treatment. Specifically, S group had a better CPC score than that of the E and NS group. Serum NSE in S group was lower than that of the E and NS groups. While CPC score was currently considered as a good scoring sys-

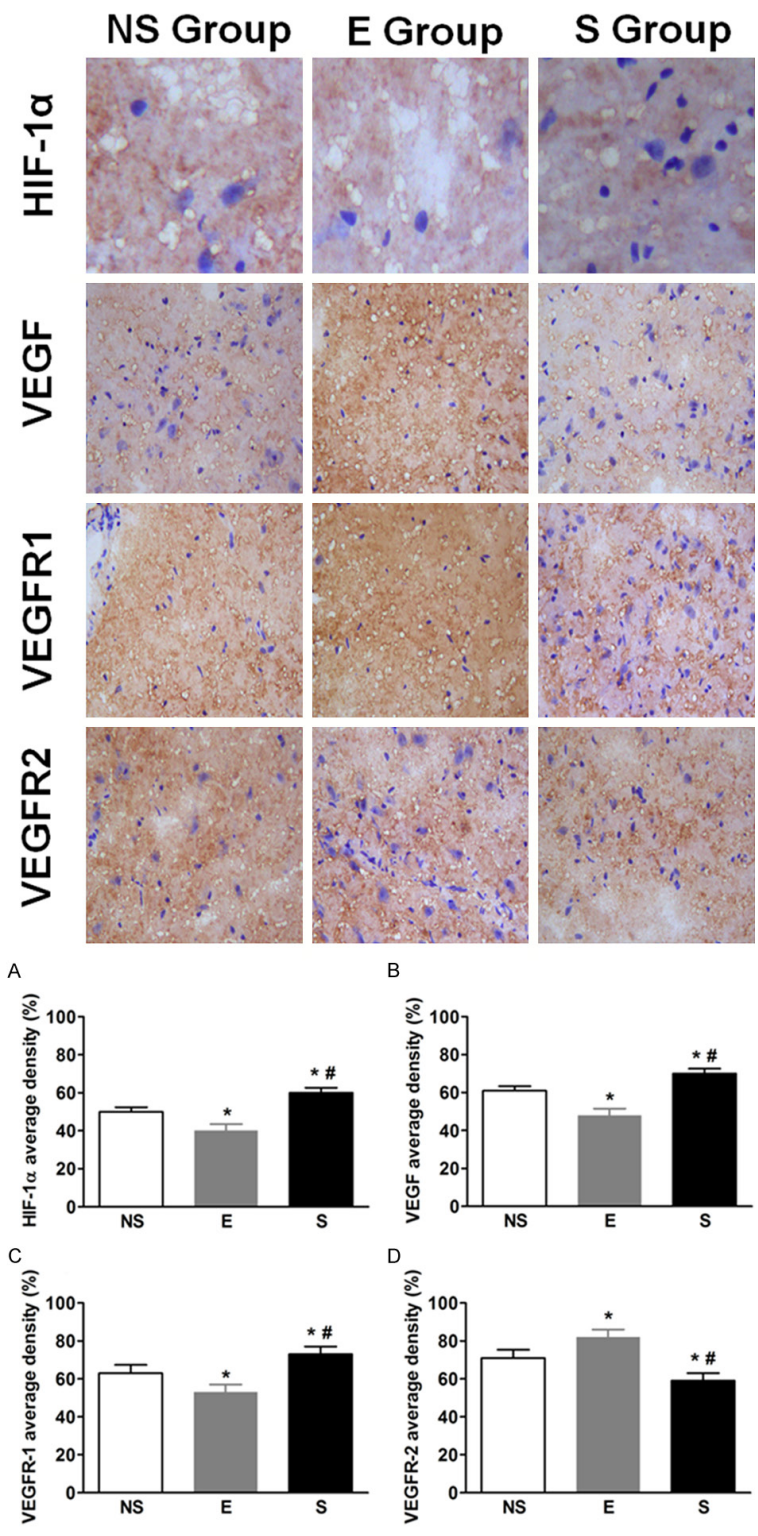

Figure 6. The post-ROSC effects of AC on the protein expression of HIF-1α, VEGF, VEGFR1, and VEGFR2 using Western blot methods. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group ($P<0.05$). *Pond* represents significant difference, as compared to E group ($P<0.05$).

tem and has been widely applied in animal experiments to evaluate neurological functions and prognosis [16], it has been proven by many clinical tests that NSE is a good indicator to predict nerve functions [17, 18]. Thus, our results consistently showed that treatment of esmolol and epinephrine after cardiac arrest may prevent nerve injuries.

Given that previous studies have shown that sudden cardiac arrest can cause partial BBB damage and the forming of serious encephaledema in the thalamus, hypothalamus or hippocampus, and cerebral cortex [19], we confirmed our neurological findings by performing histological examinations. We found that the internal structure of brain cells in the S group showed slighter damage and the organization had good integrity with less damage to BBB and lighter cellular edema, as compared with E and NS groups. It was also found that the brain water content in animals of the S group was the lowest. In contrast, the nerve damage of animals in NS group singly was slightly better than that of the E group. However, the successful rate of resuscitation was higher, the time needed for ROSC was lower, the number of defibrillation times was lower, and the survival rate within 24 hours was higher in E group, as compared with NS group. These findings are consistent with previous studies showing that treatment of epinephrine may help the ROSC but exacerbates the nerve damage [3-5]. Finally, while it has been found in previous studies that esmolol has the tendency to increase the survival rate in the early stages

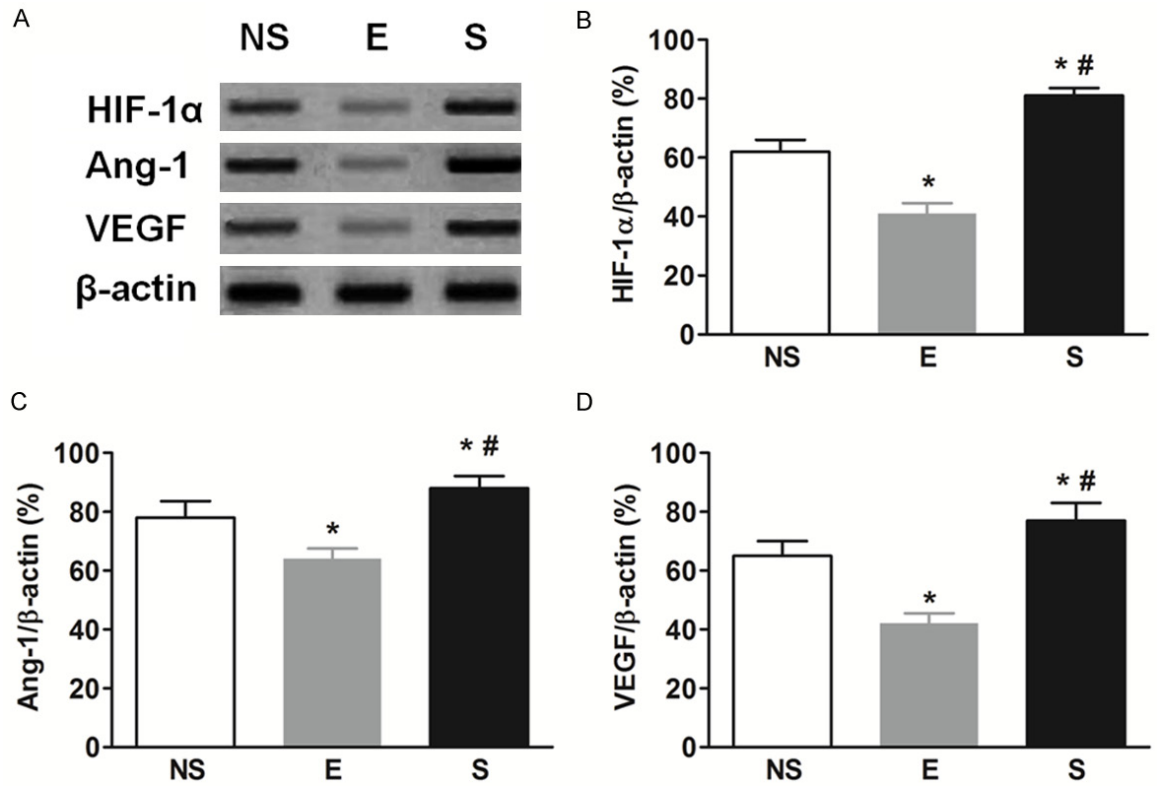

Figure 7. The post-ROSC effects of AC on the protein expression of HIF-1α, VEGF, and Ang-1 using Western blot methods. After 8 min of non-treatment operation, animals received a bolus of esmolol and epinephrine (i.e., S group; 500 μg/kg and 20 μg/kg, respectively), epinephrine only (i.e., E group; 20 μg/kg), or normal saline (i.e., NS group; 1 ml/kg), followed by standard CPR and defibrillation. *Asterisk* represents significant difference, as compared to NS group ($P<0.05$). *Pond* represents significant difference, as compared to E group ($P<0.05$).

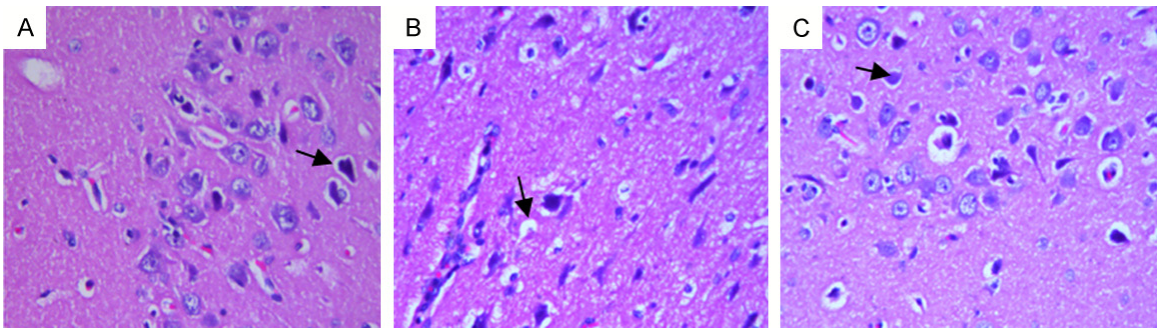

Figure 8. H&E staining of cerebral slides. 400 times magnification. (A: NS group, B: E group, C: S group). The E group's animals had the worst situation, visible neuronal necrocytosis, necrocytosis neurons weeks for visible microglia. Nerve cells reduced. It was found Occasional neuronal necrocytosis, no significant reduction in the number of nerve cells in S group and NS group.

[7, 20], our results showed that esmolol did not affect the resuscitation effect of epinephrine. Specifically, we found that esmolol and epinephrine did not prolong the time for recovering spontaneous circulation or increase the number of defibrillation times, as compared with E group. The successful rate of resuscitation for the animals between S and E groups had no difference.

In this study, we discovered that the serum concentration of HIF-1α fluctuated in the early and late stages of ROSC. In the S group, HIF-1α level did not rise in the early stages of resusci-

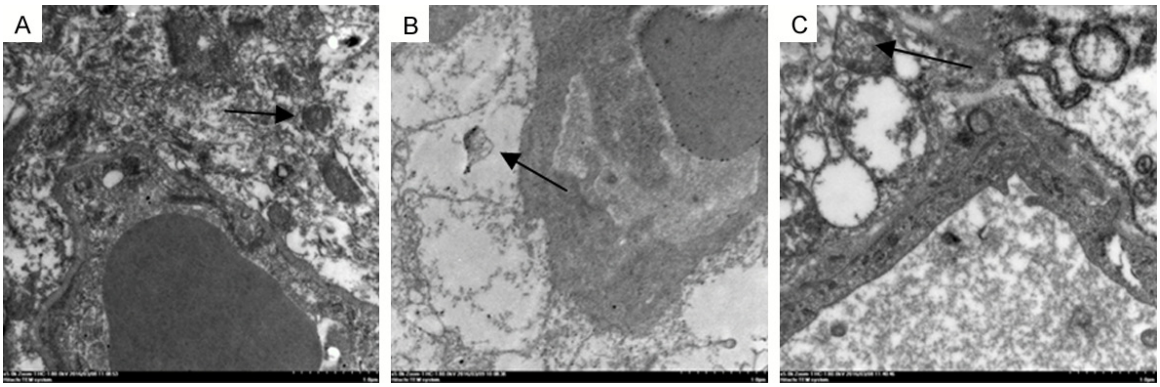

Figure 9. TEM images of cerebral slides. (A: S group, B: E group, C: NS group). Around vascular degenerated, organelles structures disappeard, vascular endothelial cell degenerated, structure was fuzzy (B). It was found swollen mitochondria in NS group, mitochondrial cristae disappear, but also could see membrane structure (C). Basic preserved vascular structures, vascular basement membrane is fuzzy. The structure of organelles basic was preserved and vascular structures keep intact, the vascular basement membrane was clear (S group).

tation, as compared with NS and E groups. However, HIF-1α level in S groups was higher than that of the other two groups of animals at 24 hours after resuscitation, which was confirmed using immunohistochemistry and Western blot. As shown in previous studies, HIF-1α is a member of the hypoxic induction factor family. It can influence the necrosis and apoptosis of nerve cells. In the early stages of ischemia, HIF-1α can up-regulate apoptosis-promoting genes [21]. Thus, the inhibition of HIF-1α by esmolol in the early stages can relieve the apoptosis of cells caused by anoxia.

Moreover, HIF-1α influences the levels of VEGF, which is the downstream factor of HIF-1α. Previous studies have shown that inhibition VEGF can reduce the permeability of BBB and reduce the risk of cerebral hemorrhage at the acute phase cerebral infarction [22]. Chen *et al.* discovered that administrations of HIF-1α inhibitor at 5 minutes, but not at 3 hours, after ischemia can reduce the levels of HIF-1α and VEGF, thereby reducing damage to BBB, relieving encephaledema, and reaching the effect of neuroprotection in an ischemic and anoxic animal model [21]. These findings suggested that early inhibition of HIF-1α and VEGF signaling may have beneficial effects on subsequent ROSC. In contrast, VEGF can increase the revascularization of ischemia brain tissue and reduce nerve damage at the recovery phase of cerebral infarction. Zhang *et al.* discovered that while a local injection of VEGF at 48 hours after cerebral ischemia can relieve BBB leakage, promote revascularization and nerve recovery, an injection of VEGF at 1 hour after ischemia caused increased BBB leakage and encephaledema [22]. These studies indicated that VEGF can improve ischemic and anoxic brain tissue functions at a later stage [13, 23, 24]. In fact, our study has shown that the levels of HIF-1α and VEGF fluctuated in the early and late stages of ROSC. Notably, the level of serum VEGF was inhibited in the early stages ROSC in the S group. Furthermore, the S group of animals was found to have high expressions of HIF-1α/VEGF and VEGFR-1 and VEGFR-2 at 24 hours after ROSC. While our studies demonstrated that esmolol and epinephrine can transiently inhibit the levels of HIF-1α/VEGF and VEGFR-1 in the early stages of ROSC, it still remains unclear the mechanisms underlying the rising levels of HIF-1α/VEGF, VEGFR-1, and VEGFR-2 in the later stages of ROSC. Therefore, future research will be necessary to explore the mechanisms underlying this phenomenon.

Our research also discovered that the levels of serum angiogenin and its receptor expression were altered during the early and late stages of ROSC. The serum levels of Ang-1 in the S group was higher than that of the E group after spontaneous circulation, and the cortical expression of Ang-1 and Tie-2 at 24 hours was also higher than that of the E group. It has been discovered that the Ang-Tie2 system not only participates in controlling angiogenesis, but also participates in regulating endothelial inflam-

matory response with VEGF and its receptors [15]. Ang-1 can neutralize the increased vascular permeability induced by VEGF by adjusting the activity of MMP-9 [25], so that the increase of Ang-1 levels may reduce the encephaledema in the S group of animals. Furthermore, Ang-1 acts on Tie-2 to stabilize endothelial cells and inhibit vascular leakage, inflammatory response, and expressions of genes that are related to thrombin [13, 26]. In contrast to Ang-1, Ang-2 is a proinflammatory factor, which increases the leakage of endothelial vessels [26]. In our study, we showed that the serum level and protein expression of Ang-2 in the S group were lower than those of the E and NS groups. In addition, the ratio of Ang-2/Ang-1 in the serum of the S group was lower than that of the E group, which indicates that its case fatality rate within 28 days is better than that of the E and NS groups. Taken together, our results indicated that esmolol and epinephrine treatment may promote the subsequent angiogenesis and inhibit endothelial inflammatory response via Ang-Tie2 system, thus reaching the effects of neuroprotection.

In conclusion, our research showed that epinephrine combined with esmololimproved neurological outcome in a pig model of CA. This phenomenon might be due to the fact that esmolol and epinephrine combination inhibited the levels of HIF-1α and VEGF in the early stages of ROSC, enhance the Ang-1 and Tie-2 signaling, thus reducing BBB leakages and relieving encephaledema.

Disclosure of conflict of interest

None.

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